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IMMUNOGENIC DETOXIFIED MUTANT *E. COLI* LT-A TOXIN

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IMMUNOGENIC DETOXIFIED MUTANT E. COLI LT-A TOXIN

Field of the invention

5   The present invention relates to immunogenic detoxified heat labile toxin proteins (LT) produced by enterotoxigenic strains of *Escherichia coli* (*E.coli*) wherein at least amino acid Ala-72 of the A subunit is mutated, and to their use in vaccines which are useful in the prevention or treatment of

10   enterotoxigenic *E.coli* infections and as adjuvants for other immunogenic proteins. Toxoids of the invention can be suitably produced using recombinant DNA techniques by site-directed mutagenesis of DNA encoding the wild-type toxins.

15

Background to the Invention

Heat-labile enterotoxin (LT), produced by enterotoxigenic strains of *E.coli* , and cholera toxin (CT), produced by

20   *V.cholerae* strains, are the causative agents of traveller's diarrhoea and cholera, respectively [Spangler (1992) *Microbiol Rev* 56:622]. LT and CT show 80% homology in the primary structure and an identical tertiary structure. They are composed of two functionally distinct domains: the

25   enzymatically-active A subunit and the B pentamer, which contains the receptor-binding site. The A subunit ADP-ribosylates the target protein Gs, a GTP-binding protein which regulates the intracellular levels of cAMP [Rappuoli & Pizza (1991), in *Sourcebook of bacterial protein toxins*,

30   Academic Press NY]. Enhancement in cAMP levels can alter ion transport, inducing secretion of water and chloride ions into the intestine.

CT and LT are both powerful immunogens and potent mucosal

35   adjuvants when co-administered with antigens at the mucosal level [eg. Jackson et al. (1993) *Infect Immun* 61:4272; WO95/17211]. Immunogenicity and adjuvanticity of wild-type CT and LT have been extensively studied in animals [eg.

Rollwagen *et al.* (1993) *Vaccine* 11:1316], but their toxicity
has precluded their use in humans. In an attempt to overcome
the problems generated by the use of active holotoxins, two
different approaches have been followed, one based on the
5 use of the B pentamer, the non-toxic domain of the holotoxin
[*eg.* Holmgren *et al.* (1992) *Vaccine*10:911], and the other
based on the generation of genetically detoxified
derivatives of LT and CT [*eg.* Fontana *et al.* (1995) *Infect
Immun* 63:2356]. Site-directed mutagenesis on both A and B
10 subunits has provided a tool to explore the basis of the
immunological and adjuvant responses induced by these
molecules.

Examples of such experiments can be found in:
15 - Harford *et al.* [*Eur J Biochem* (1989) 183:311] made LT-A
carrying Ser-61-Phe and Gly-79-Lys substitutions.
- Tsuji *et al.* [*J Biol Chem* (1990) 265:22520] produced LT-A
carrying a Glu-112-Lys substitution.
- Burnette *et al.* [*Infect Immun* (1991) 59:4266] produced
20 CT-A carrying a Arg-7-Lys substitution. This work can also
be seen in WO92/19265.
- WO93/13202 described non-toxic CT and LT carrying
mutations at Val-53, Ser-63, Val-97, Tyr-104, and Pro-106

25 A mutant in the receptor binding site of the B subunit of
LT, the G33D mutant, has been reported to lack the
immunological properties of the native B subunit [Nashar *et
al.* (1996) *PNAS* 93:226], suggesting that binding to the
receptor is important for the immunogenicity. It has also
30 been shown that non-toxic derivatives of LT carrying mutant
A subunits retain the immunological properties of wild-type
LT [*eg.* Magagnoli *et al.* (1996) 64:5434], suggesting that
ADP-ribosylation activity is not essential for
immunogenicity.
35

In relation to adjuvanticity, much data has been generated
but many questions remain unanswered. Some studies have
suggested that LT-B and CT-B have adjuvant activity, but the

conclusions drawn have been compromised by contamination
with active toxin [Wilson *et al.* (1993) *Vaccine* 11:113].
Studies with recombinant LT-B and CT-B have suggested that
they behave as poor mucosal adjuvants [*eg.* Douce *et al.*
5   (1997) *Infect. Immun.* 65:2821]

Attempts to define the role of ADP-ribosylation activity in
the adjuvanticity of LT has generated conflicting results.
Lycke *et al.* [*Eur J Immunol* (1992) 22:2277] have described
10 a   non-toxic   LT   derivative   (LT-E112K)   which,   when
administered with KLH by oral route in mice, lacked the
adjuvant properties of wild-type LT, thus suggesting that
adjuvant activity is linked to ADP-ribosylation activity. LT
derivatives such as LT-K7 and LT-K63 [*eg.* Douce *et al.*
15 (1997) *supra;* Douce *et al.* (1996) *PNAS* 92:1644; DiTommaso
et al. (1996) *Infect Immun* 64:974], however, which are
devoid of toxicity both *in vitro* and *in vivo*, have been
shown to be able to elicit an antibody response against a
co-administered antigen in intranasally immunised mice.
20 LT-K63 has been shown to induce measle-specific CTL response
after intranasal immunisation with a synthetic peptide
[Partidos *et al.* (1996) *Immunol* 89:483], and strongly
enhances protection against *H.pylori* following intragastric
immunisation with *H.pylori* antigens. The antibody titres
25 induced by these non-toxic LT mutants were lower than those
obtained with wild-type toxin, however, and were only
detected after two mucosal immunisations [Douce *et al.*
(1997) *supra*].

30 It is an object of the invention to provide forms of LT
which are detoxified, so that they might be suitable for use
in humans, but which retain the adjuvant and immunogenic
properties of LT as far as possible.

35 <u>Summary of the invention</u>

According to a first aspect of the present invention, there
is provided an immunogenic detoxified protein comprising the

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amino acid sequence of subunit A of an *E.coli* heat labile
toxin (LT-A), or a fragment thereof, wherein at least amino
acid Ala-72 in said sequence or fragment is mutated.

5   As used herein, the term "detoxified" means that the toxoid
exhibits a reduction in toxicity relative to the wild-type
toxin. The toxicity may be measured in mouse cells, CHO
cells, by evaluation of the morphological changes induced in
Y1 cells, or preferably by the rabbit ileal loop assay. As
10 measured in Y1 cells, for instance, "detoxified" means that
the toxoid exhibits a reduction in toxicity relative to the
wild-type toxin of greater than 10,000-fold.

Any residual toxicity should be sufficiently low for the
15 protein to be used in an effective immunogenic composition
without causing significant side effects. Certain mutants
within the scope of the invention may possess zero toxic
activity.

20 As used herein, the term "residual toxicity" means that the
detoxified immunogenic protein may retain a measurable
toxicity. More particularly the level of toxicity may be
optimised in a benefit/side-effect trade-off to maximise
immunogenicity and/or adjuvanticity whilst maintaining a
25 sufficiently low toxicity to be tolerated by the subject
after administration.

Thus, although these proteins are detoxified in the sense of
having a much lower toxicity than the wild-type protein,
30 traces of the enzymatic activity responsible for toxicity
may remain. The mutation causes a decrease in toxicity of
which makes the toxoid suitable for human use.

Most preferably the toxicity of the toxoid is reduc d
35 relative to its natural occurring counterpart by greater
than 10,000 fold as measured by the evaluation of the
morphological changes induced in Y1 cells or greater than 10
fold as measured by the rabbit ileal loop assay, perform d

as described herein.

The term "toxoid" as used herein means a detoxified mutated toxin protein.

5

In this specification, references to LT encompass the various naturally occurring strain variants as well other variants encompassing mutations which do not significantly alter the properties of the subunits or of the assembled
10 holotoxin. These mutations may be, for instance, conservative amino acid changes which do not affect the assembly of the holotoxin.

References to LT-A also encompass fragments of LT-A provided
15 that the fragment contains Ala-72.

Most importantly, the toxoid must remain immunologically cross-reactive with the toxin from which it is derived.

20 The immunogenic protein may be LT subunit A toxoid, but is preferably an assembled holotoxoid comprising a LT-A toxoid and five LT-B subunits, which may themselves be wild-type or mutated.

25 It will be appreciated that in derivatives of LT-A, such as fragments, or in LT-A proteins of different *E.coli* strains, the amino acid residue to be mutated is that which corresponds to Ala-72 as defined for LT-A in Domenighini *et al.* [*Molec. Microbiol.* (1995) 15:1165-1167]. Ala-72 is
30 located on the second turn of the alpha-helix in LT-A and faces the NAD binding site.

The mutation at Ala-72 may be a substitution, an insertion, or a deletion. Preferably it is a substitution with a
35 different amino acid. The most preferred mutation is the substitution of Ala-72 with arginine, for which the standard nomenclatur is A72R.

Whilst the A72R mutant retains residual toxicity, other
mutants within the scope of the invention may possess no
toxicity, for example mutants with one or more mutations at
other sites, or toxoids which are further detoxified by
5 chemical means.

The or each amino acid substituted for a wild type amino
acid, whether at Ala-72 or elsewhere, may be a naturally
occurring amino acid or may be a modified or synthetic amino
10 acid, provided that the mutant retains the necessary
immunogenic and detoxified properties.

Substitutions which alter the amphotericity and/or
hydrophilicity of the protein whilst retaining as far as
15 possible the steric effect of the substituted amino acid are
generally preferred.

The toxoids of the invention may be synthesised chemically
using conventional peptide synthesis techniques, but are
20 preferably produced by recombinant DNA means.

Preferably the toxoid is obtained in substantially pure
form.

25 According to a second aspect of the invention, there is
provided an immunogenic composition comprising an
immunogenic detoxified protein of the first aspect of the
invention and a pharmaceutically acceptable carrier. This
immunogenic composition may be a vaccine against the
30 enterotoxigenic strains of E.coli itself and may thus
optionally further comprise an adjuvant.

In addition to the properties typical of mutants devoid of
enzymatic activity, the mutant proteins also exhibit
35 adjuvant activity.

Additionally, the immunogenic composition may further
comprise a second antigen capable of raising an

immunological response to another disease. In such an alternative composition, the immunogenic detoxified protein of the invention can act as a mucosal adjuvant.

5   According to a third aspect of the invention, there is provided the use of a toxoid of the first aspect as an adjuvant.

According to a fourth aspect of the invention, there is
10 provided a method of vaccinating a mammal against an enterotoxigenic strain of *E.coli* comprising administering an immunologically effective amount of an immunogenic detoxified protein according to the first aspect of the invention. Optionally, the immunogenic detoxified protein of
15 the invention may act as an adjuvant for a second immunogenic protein administered separately, sequentially or with the immunogenic detoxified protein of the invention.

According to a fifth aspect of the invention, there is
20 provided a DNA sequence encoding an immunogenic detoxified protein according to the first aspect of the invention.

Preferably the DNA sequence encodes both the detoxified subunit A and subunit B in a polycistronic unit.
25 Alternatively, the DNA may encode only the detoxified subunit A.

According to a sixth aspect of the invention, there is provided a vector comprising a DNA sequence according to the
30 fifth aspect of the invention.

According to a seventh aspect of the invention, there is provided a host cell transformed with a vector according to the sixth aspect of the invention.
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The host cell may be any host capable of expressing a DNA sequence according to the fifth aspect, but is preferably a bacterium, most preferably *E.coli*, suitably transfected to

produce the desired immunogenic detoxified protein.

In a further embodiment of the seventh aspect of the invention, the host cell may itself provide a protective
5 species, for example an *E.coli* strain mutated to a phenotype lacking wild type LT and expressing an immunogenic detoxified protein of the first aspect of the invention.

According to a eighth aspect of the invention, there is
10 provided a process for the production of an immunogenic detoxified protein according to the first aspect of the invention comprising culturing a host cell according to the seventh aspect of the invention.

15 According to a ninth aspect of the invention, there is provided a process for the production of DNA according to the fifth aspect of the invention comprising the steps of subjecting a DNA encoding a wild-type LT-A or a fragment thereof to site-directed mutagenesis.
20
According to a tenth aspect of the invention, there is provided a process for the formulation of an immunogenic composition according to the second aspect' comprising bringing an immunogenic detoxified protein according to the
25 first aspect of the invention into association with a pharmaceutically acceptable carrier, and optionally with an adjuvant.

According to a eleventh aspect of the invention, there is
30 provided a method for the prevention or treatment of disease in a subject, comprising administering to said subject an immunologically effective dose of an immunogenic composition according to the second aspect.

35 Industrial Applicability

The immunogenic detoxified protein of the invention may constitute the active component of a vaccine composition

useful for the prevention and treatment of infections by enterotoxigenic strains of *E.coli*. The immunogenic detoxified protein may also be used in a vaccine composition as a mucosal adjuvant. The compositions are thus applicable
5   for use in the pharmaceutical industry.

Description of the drawings

Figures 1 to 5 show the biochemical and biological
10   properties of wild-type LT and of mutants LT-A72R (also shown as "LTR72") and LTK63: **Figure 1** is a chromatographic profile on a superdex column, with peak I corresponding to holotoxin and peak II to the EDTA in the buffer; **Figure 2** is a Western blot of a trypsin digest experiment; **Figure 3**
15   shows the reults of *in vitro* ADP-ribosylation of polyarginine; and **Figures 4 and 5** show the results of *in vitro* and *in vivo* toxicity experiments, respectively.

Figure 6 shows serum anti-OVA antibody response after three
20   intranasal immunisations (indicated by arrows). **Figure 7** shows the IgG subclasses after the third immunisation. **Figure 8** shows the IgA levels after the third immunisation in serum samples (8A) and nasal washes (8B) [mean titre and standard deviation are shown].
25
Figure 9 shows serum anti-LT Ig (9A) and IgA (9B) responses.

Figure 10 shows OVA-driven proliferative responses. Background values (no OVA added to clutures) varied between
30   1000 and 3000 cpm.

Figure 11 shows the efficacy against systemic challenge of intranasal immunisation with wild-type LT and with mutants A72R and K63. Columns in 11B are mean titres and dots are
35   individual titres. Black dots show titres from mice immunised with LTB and not protected against challenge.

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Detailed Description of Embodiments of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular
5   biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See eg., Sambrook, et al., MOLECULAR CLONING; A LABORATORY MANUAL, SECOND EDITION (1989); DNA CLONING, VOLUMES I AND II (D.N Glover ed. 1985);
10   OLIGONUCLEOTIDE SYNTHESIS (M.J. Gait ed, 1984); NUCLEIC ACID HYBRIDIZATION (B.D. Hames & S.J. Higgins eds. 1984); TRANSCRIPTION AND TRANSLATION (B.D. Hames & S.J. Higgins eds. 1984); ANIMAL CELL CULTURE (R.I. Freshney ed. 1986); IMMOBILIZED CELLS AND ENZYMES (IRL Press, 1986); B. Perbal,
15   A PRACTICAL GUIDE TO MOLECULAR CLONING (1984); the series, METHODS IN ENZYMOLOGY (Academic Press, Inc.); GENE TRANSFER VECTORS FOR MAMMALIAN CELLS (J.H. Miller and M.P. Calos eds. 1987, Cold Spring Harbor Laboratory), Methods in Enzymology Vol. 154 and Vol. 155 (Wu and Grossman, and Wu, eds.,
20   respectively), Mayer and Walker, eds. (1987), IMMUNOCHEMICAL METHODS IN CELL AND MOLECULAR BIOLOGY (Academic Press, London), Scopes, (1987), PROTEIN PURIFICATION: PRINCIPLES AND PRACTICE, Second Edition (Springer-Verlag, N.Y.), and HANDBOOK OF EXPERIMENTAL IMMUNOLOGY, VOLUMES I-IV (D.M. Weir
25   and C. C. Blackwell eds 1986).

Standard abbreviations for nucleotides and amino acids are used in this specification. All publications ,patents, and patent applications cited herein are incorporated by
30   reference.

In particular, the following amino acid abbreviations are used:

35	Alanine	A	Ala	Arginine	R	Arg
	Asparagine	N	Asn	Aspartic Acid	D	Asp
	Cysteine	C	Cys	Glycine	G	Gly
	Glutamic Acid	E	Glu	Glutamine	Q	Gln

Histidine	H	His	Isoleucine	I	Ile
Leucine	L	Leu	Lysine	K	Lys
Methionine	M	Met	Phenylalanine	F	Phe
Proline	P	Pro	Serine	S	Ser
Threonine	T	Thr	Tryptophan	W	Trp
Tyrosine	Y	Tyr	Valine	V	Val

As mentioned above examples of the immunogenic detoxified protein that can be used in the present invention include polypeptides with minor amino acid variations from the natural amino acid sequence of the protein other than at the sites of mutation specifically mentioned.

A significant advantage of producing the immunogenic detoxified protein by recombinant DNA techniques rather than by isolating and purifying a protein from natural sources is that equivalent quantities of the protein can be produced by using less starting material than would be required for isolating the protein from a natural source. Producing the protein by recombinant techniques also permits the protein to be isolated in the absence of some molecules normally present in cells. Indeed, protein compositions entirely free of any trace of human protein contaminants can readily be produced because the only human protein produced by the recombinant non-human host is the recombinant protein at issue. Potential viral agents from natural sources and viral components pathogenic to humans are also avoided. Also, genetically detoxified toxin are less likely to revert to a toxic from than more traditional, chemically detoxified toxins.

Pharmaceutically acceptable carriers include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplets or

liposomes) and inactive virus particles. Such carriers are
well known to those of ordinary skill in the art.
Additionally, these carriers may function as
immunostimulating agents (adjuvants).

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Preferred aditional adjuvants to enhance effectiveness of
immunogenic compositions include, but are not limited to:
aluminum salts (alum) such as aluminium hydroxide, aluminium
phosphate, aluminium sulfate *etc*, oil emulsion formulations,
10 with or without other specific immunostimulating agents such
as muramyl peptides or bacterial cell wall components, such
as for example (1) MF59 (Published International patent
application WO90/14837, containing 5% Squalene, 0.5% Tween®
80, 0.5% Span® 85 (optionally containing various amounts of
15 MTP-PE (see below), although not required) formulated into
submicron particles using a microfluidizer such as Model
110Y microfluidizer (Microfluidics, Newton, MA 02164), (2)
SAF, containing 10% squalene, 0.4% Tween 80, 5% pluronic-
blocked polymer L121, and thr-MDP (see below) either
20 microfluidized into a submicron emulsion or vortexed to
generate a larger particle size emulsion, and (3) RIBI™
adjuvant system (RAS) (Ribi Immunochem, Hamilton, MT)
containing 2% Squalene, 0.2% Tween® 80 and one or more
bacterial cell wall components from the group consisting of
25 monophosphoryl lipid A (MPL), trehalose dimycolate (TDM),
and cell wall skeleton (CWS) preferably MPL+CWS (Detox™),
muramyl peptides such as N-acetyl-muramyl-L-threonyl-D-
isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-iso-
glutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-
30 isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-*sn*-glycero-3-
hydroxyphosphoryloxy)-ethylamine (MTP-PE) etc., and
cytokines, such as interleukins (IL-1, IL-2 etc) macrophage
colony stimulating factor (M-CSF), tumour necrosis factor
(TNF) *etc*. Additionally, saponin adjuvants, such as
35 Stimulon™ (Cambridge Bioscience, Worcester, MA) may be used
or particles generated therefrom such as ISCOMS
(immunostimulating complexes). Furthermore, Complete Freunds
Adjuvant (CFA) and Incomplete Freunds Adjuvant (IFA) may be

used. Alum and MF59 are preferred.

The immunogenic detoxified protein of the invention may be used as an adjuvant for a second antigen in an immunologically active composition for the treatment or vaccination of the human or animal body.

The immunogenic compositions (eg the antigen, pharmaceutically acceptable carrier and adjuvant) typically will contain diluents, such as water, saline, glycerol, ethanol, etc. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles.

Typically, the immunogenic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. The preparation also may be emulsified or encapsulated in liposomes for enhanced adjuvant effect as discussed above under pharmaceutically acceptable carriers.

Immunogenic compositions used as vaccines comprise an immunologically effective amount of the antigenic polypeptides, as well as any other of the above-mentioned components, as needed. By "immunologically effective amount", it is meant that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment or prevention. This amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated (eg., nonhuman primate, primate, etc.), the capacity of the individual's immune system to synthesize antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through

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routine trials.

The immunogenic compositions are conventionally administered
parenterally, *eg.* by injection either subcutaneously or
5   intramuscularly. Additional formulations suitable for other
modes of administration include oral and pulmonary
formulations, suppositories and transdermal applications.
Dosage treatment may be a single dose schedule or a multiple
dose schedule. The vaccine may be administered in conjunc-
10 tion with other immunoregulatory agents.

The term "recombinant polynucleotide" as used herein intends
a polynucleotide of genomic, cDNA, semisynthetic, or
synthetic origin which, by virtue of its origin or
15 manipulation: (1) is not associated with all or a portion of
a polynucleotide with which it is associated in nature, (2)
is linked to a polynucleotide other than that to which it is
linked in nature, or (3) does not occur in nature.

20 The term "polynucleotide" as used herein refers to a
polymeric form of nucleotides of any length, either
ribonucleotides or deoxyribonucleotides. This term refers
only to the primary structure of the molecule. Thus, this
term includes double- and single-stranded DNA and RNA. It
25 also includes known types of modifications, for example,
labels which are known in the art, methylation, "caps",
substitution of one or more of the naturally occurring
nucleotides with an analog, internucleotide modifications
such as, for example, those with uncharged linkages (*eg.*,
30 methyl phosphonates, phosphotriesters, phosphoamidates,
carbamates, *etc.*) and with charged linkages (*eg.*,
phosphorothioates, phosphorodithioates, *etc.*), those
containing pendant moieties, such as, for example proteins
(including for *eg.*, nucleases, toxins, antibodies, signal
35 peptides, poly-L-lysine, etc.), those with intercalators
(*g.*, acridine, psoralen, etc.), those containing chelators
(*eg.*, metals, radioactive metals, boron, oxidative metals,
etc.), those containing alkylators, those with modified

linkages (*eg.*, alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide.

A "replicon" is any genetic element, *eg.*, a plasmid, a
5   chromosome, a virus, a cosmid, etc. that behaves as an autonomous unit of polynucleotide replication within a cell; i.e., capable of replication under its own control. This may include selectable markers.

10   A "vector" is a replicon in which another polynucleotide segment is attached, so as to bring about the replication and/or expression of the attached segment.

"Control sequence" refers to polynucleotide sequences which
15   are necessary to effect the expression of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism; in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and transcription
20   termination sequence; in eukaryotes, generally, such control sequences include promoters and transcription termination sequence. The term "control sequences" is intended to include, at a minimum, all components whose presence is necessary for expression, and may also include additional
25   components whose presence is advantageous, for example, leader sequences and fusion partner sequences.

"Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to
30   function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

35

An "open reading frame" (ORF) is a region of a polynucleotide sequence which encodes a polypeptide; this region may represent a portion of a coding sequnce or a

total coding sequence.

A "coding sequence" is a polynucleotide sequence which is
translated into a polypeptide, usually via mRNA, when placed
5 under the control of appropriate regulatory sequences. The
boundaries of the coding sequence are determined by a
translation start codon at the 5'-terminus and a translation
stop codon at the 3'-terminus. A coding sequence can
include, but is not limited to, cDNA, and recombinant
10 polynucleotide sequences.

"PCR" refers to the technique of polymerase chain reaction
as described in Saiki, et al., Nature 324:163 (1986); and
Scharf et al., Science (1986) 233:1076-1078; and U.S.
15 4,683,195; and U.S. 4,683,202.

As used herein, x is "heterologous" with respect to y if x
is not naturally associated with y in the identical manner;
i.e., x is not associated with y in nature or x is not
20 associated with y in the same manner as is found in nature.

"Homology" refers to the degree of similarity between x and
y. The correspondence between the sequence from one form to
another can be determined by techniques known in the art.
25 For example, they can be determined by a direct comparison
of the sequence information of the polynucleotide.
Alternatively, homology can be determined by hybridization
of the polynucleotides under conditions which form stable
duplexes between homologous regions (for example, those
30 which would be used prior to S_1 digestion), followed by
digestion with single-stranded specific nuclease(s), fol-
lowed by size determination of the digested fragments.

As used herein, the term "polypeptide" refers to a polymer
35 of amino acids and does not refer to a specific length of
the product; thus, peptides, oligopeptides, and proteins are
included within the definition of polypeptide. This term
also does not refer to or exclude post expression

modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. Included within the definition are, for example, polypeptides containing one or more analogs of an amino acid
5   (including, for example, unnatural amino acids, etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

10   A polypeptide or amino acid sequence "derived from" a designated nucleic acid sequence refers to a polypeptide having an amino acid sequence identical to that of a polypeptide encoded in the sequence, or a portion thereof wherein the portion consists of at least 3-5 amino acids,
15   and more preferably at least 8-10 amino acids, and even more preferably at least 11-15 amino acids, or which is im- munologically identifiable with a polypeptide encoded in the sequence. This terminology also includes a polypeptide expressed from a designated nucleic acid sequence.
20
The protein may be used for producing antibodies, either monoclonal or polyclonal, specific to the protein. The methods for producing these antibodies are known in the art.

25   "Recombinant host cells", "host cells," "cells," "cell cultures," and other such terms denote, for example, microorganisms, insect cells, and mammalian cells, that can be, or have been, used as recipients for recombinant vector or other transfer DNA, and include the progeny of the
30   original cell which has been transformed. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation. Examples for
35   mammalian host cells include Chinese hamster ovary (CHO) and monkey kidney (COS) cells.

Specifically, as used herein, "cell line," refers to a

population of cells capable of continuous or prolonged
growth and division <u>in vitro</u>. Often, cell lines are clonal
populations derived from a single progenitor cell. It is
further known in the art that spontaneous or induced changes
5   can occur in karyotype during storage or transfer of such
clonal populations. Therefore, cells derived from the cell
line referred to may not be precisely identical to the
ancestral cells or cultures, and the cell line referred to
includes such variants. The term "cell lines" also includes
10   immortalized cells. Preferably, cell lines include nonhybrid
cell lines or hybridomas to only two cell types.

As used herein, the term "microorganism" includes
prokaryotic and eukaryotic microbial species such as
15   bacteria and fungi, the latter including yeast and
filamentous fungi.

"Transformation", as used herein, refers to the insertion of
an exogenous polynucleotide into a host cell, irrespective
20   of the method used for the insertion, for example, direct
uptake, transduction, f-mating or electroporation. The
exogenous polynucleotide may be maintained as a
non-integrated vector, for example, a plasmid, or
alternatively, may be integrated into the host genome.
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By "genomic" is meant a collection or library of DNA
molecules which are derived from restriction fragments that
have been cloned in vectors. This may include all or part of
the genetic material of an organism.
30

By "cDNA" is meant a complementary DNA sequence that
hybridizes to a complementary strand of DNA.

By "purified" and "isolated" is meant, when referring to a
35   polypeptide or nucleotide sequence, that the indicated
molecule is present in the substantial absence of other
biological macromolecules of the same type. The term
"purified" as used herein preferably means at least 75% by

weight, more preferably at least 85% by weight, more
preferably still at least 95% by weight, and most preferably
at least 98% by weight, of biological macromolecules of the
same type present (but water, buffers, and other small
5   molecules, especially molecules having a molecular weight of
less than 1000, can be present).

Once the appropriate coding sequence is isolated, it can be
expressed in a variety of different expression systems; for
10 example those used with mammalian cells, baculoviruses,
bacteria, and yeast.

i. Mammalian Systems

15 Mammalian expression systems are known in the art. A
mammalian promoter is any DNA sequence capable of binding
mammalian RNA polymerase and initiating the downstream (3')
transcription of a coding sequence (eg. structural gene)
into mRNA. A promoter will have a transcription initiating
20 region, which is usually placed proximal to the 5' end of
the coding sequence, and a TATA box, usually located 25-30
base pairs (bp) upstream of the transcription initiation
site. The TATA box is thought to direct RNA polymerase II to
begin RNA synthesis at the correct site. A mammalian
25 promoter will also contain an upstream promoter element,
usually located within 100 to 200 bp upstream of the TATA
box. An upstream promoter element determines the rate at
which transcription is initiated and can act in either
orientation [Sambrook et al. (1989) "Expression of Cloned
30 Genes in Mammalian Cells." In Molecular Cloning: A
Laboratory Manual, 2nd ed.].

Mammalian viral genes are often highly expressed and have a
broad host range; therefore sequences encoding mammalian
35 viral genes provid particularly useful promoter sequences.
Examples include the SV40 early promoter, mouse mammary
tumor virus LTR promoter, adenovirus major late promoter (Ad
MLP), and herpes simplex virus promoter. In addition,

sequences derived from non-viral genes, such as the murine metallotheionein gene, also provide useful promoter sequences. Expression may be either constitutive or regulated (inducible), depending on the promoter can be
5   induced with glucocorticoid in hormone-responsive cells.

The presence of an enhancer element (enhancer), combined with the promoter elements described above, will usually increase expression levels. An enhancer is a regulatory DNA
10 sequence that can stimulate transcription up to 1000-fold when linked to homologous or heterologous promoters, with synthesis beginning at the normal RNA start site. Enhancers are also active when they are placed upstream or downstream from the transcription initiation site, in either normal or
15 flipped orientation, or at a distance of more than 1000 nucleotides from the promoter [Maniatis et al. (1987) Science 236:1237; Alberts et al. (1989) Molecular Biology of the Cell, 2nd ed.]. Enhancer elements derived from viruses may be particularly useful, because they usually have a
20 broader host range. Examples include the SV40 early gene enhancer [Dijkema et al (1985) EMBO J. 4:761] and the enhancer/promoters derived from the long terminal repeat (LTR) of the Rous Sarcoma Virus [Gorman et al. (1982b) Proc. Natl. Acad. Sci. 79:6777] and from human cytomegalovirus
25 [Boshart et al. (1985) Cell 41:521]. Additionally, some enhancers are regulatable and become active only in the presence of an inducer, such as a hormone or metal ion [Sassone-Corsi and Borelli (1986) Trends Genet. 2:215; Maniatis et al. (1987) Science 236:1237].
30

A DNA molecule may be expressed intracellularly in mammalian cells. A promoter sequence may be directly linked with the DNA molecule, in which case the first amino acid at the N-terminus of the recombinant protein will always be a
35 methionine, which is encoded by the ATG start codon. If desired, the N-terminus may be cleaved from the protein by in vitro incubation with cyanogen bromide.

Alternatively, foreign proteins can also be secreted from
the cell into the growth media by creating chimeric DNA
molecules that encode a fusion protein comprised of a leader
sequence fragment that provides for secretion of the foreign
5 protein in mammalian cells. Preferably, there are processing
sites encoded between the leader fragment and the foreign
gene that can be cleaved either in vivo or in vitro. The
leader sequence fragment usually encodes a signal peptide
comprised of hydrophobic amino acids which direct the
10 secretion of the protein from the cell. The adenovirus
triparite leader is an example of a leader sequence that
provides for secretion of a foreign protein in mammalian
cells.

15 Usually, transcription termination and polyadenylation
sequences recognized by mammalian cells are regulatory
regions located 3' to the translation stop codon and thus,
together with the promoter elements, flank the coding
sequence. The 3' terminus of the mature mRNA is formed by
20 site-specific post-transcriptional cleavage and polya-
denylation [Birnstiel et al. (1985) Cell 41:349; Proudfoot
and Whitelaw (1988) "Termination and 3' end processing of
eukaryotic RNA. In Transcription and splicing (ed. B.D.
Hames and D.M. Glover); Proudfoot (1989) Trends Biochem.
25 Sci. 14:105]. These sequences direct the transcription of an
mRNA which can be translated into the polypeptide encoded by
the DNA. Examples of transcription
terminater/polyadenylation signals include those derived
from SV40 [Sambrook et al (1989) "Expression of cloned genes
30 in cultured mammalian cells." In Molecular Cloning: A
Laboratory Manual].

Some genes may be expressed more efficiently when introns
(also called intervening sequences) are present. Several
35 cDNAs, however, have been efficiently expressed from vectors
that lack splicing signals (also called splice donor and
acceptor sites) [see eg., Gothing and Sambrook (1981) Nature
293:620]. Introns are intervening noncoding sequences within

a coding sequence that contain splice donor and acceptor sites. They are removed by a process called "splicing," following polyadenylation of the primary transcript [Nevins (1983) _Annu. Rev. Biochem. 52_:441; Green (1986) _Annu. Rev._

5 _Genet. 20_:671; Padgett et al. (1986) _Annu. Rev. Biochem. 55_:1119; Krainer and Maniatis (1988) "RNA splicing." In _Transcription and splicing_ (ed. B.D. Hames and D.M. Glover)].

10 Usually, the above described components, comprising a promoter, polyadenylation signal, and transcription termination sequence are put together into expression constructs. Enhancers, introns with functional splice donor and acceptor sites, and leader sequences may also be

15 included in an expression construct, if desired. Expression constructs are often maintained in a replicon, such as an extrachromosomal element (_eg._, plasmids) capable of stable maintenance in a host, such as mammalian cells or bacteria. Mammalian replication systems include those derived from

20 animal viruses, which require trans-acting factors to replicate. For example, plasmids containing the replication systems of papovaviruses, such as SV40 [Gluzman (1981) _Cell 23_:175] or polyomavirus, replicate to extremely high copy number in the presence of the appropriate viral T antigen.

25 Additional examples of mammalian replicons include those derived from bovine papillomavirus and Epstein-Barr virus. Additionally, the replicon may have two replication systems, thus allowing it to be maintained, for example, in mammalian cells for expression and in a procaryotic host for cloning

30 and amplification. Examples of such mammalian-bacteria shuttle vectors include pMT2 [Kaufman et al. (1989) _Mol. Cell. Biol. 9_:946 and pHEBO [Shimizu et al. (1986) _Mol. Cell. Biol. 6_:1074].

35 The transformation procedure used depends upon the host to be transformed. Methods for introduction of heterologous polynucleotides into mammalian cells are known in the art and include dextran-mediated transfection, calcium phosphate

precipitation, polybrene mediated transfection, protoplast
fusion, electroporation, encapsulation of the
polynucleotide(s) in liposomes, and direct microinjection of
the DNA into nuclei.

Mammalian cell lines available as hosts for expression are
known in the art and include many immortalized cell lines
available from the American Type Culture Collection (ATCC),
including but not limited to, Chinese hamster ovary (CHO)
cells, HeLa cells, baby hamster kidney (BHK) cells, monkey
kidney cells (COS), human hepatocellular carcinoma cells
(*eg.*, Hep G2), and a number of other cell lines.

ii. Baculovirus Systems
The polynucleotide encoding the protein can also be inserted
into a suitable insect expression vector, and is operably
linked to the control elements within that vector. Vector
construction employs techniques which are known in the art.

Generally, the components of the expression system include
a transfer vector, usually a bacterial plasmid, which
contains both a fragment of the baculovirus genome, and a
convenient restriction site for insertion of the
heterologous gene or genes to be expressed; a wild type
baculovirus with a sequence homologous to the baculovirus-
specific fragment in the transfer vector (this allows for
the homologous recombination of the heterologous gene in to
the baculovirus genome); and appropriate insect host cells
and growth media.

After inserting the DNA sequence encoding the protein into
the transfer vector, the vector and the wild type viral
genome are transfected into an insect host cell where the
vector and viral genome are allowed to recombine. The
packaged recombinant virus is expressed and recombinant
plaques are identified and purified. Materials and methods
for baculovirus/insect cell expression systems are
commercially available in kit form fr m, *inter alia*,

Invitrogen, San Diego CA ("MaxBac" kit). These techniques
are generally known to those skilled in the art and fully
described in Summers and Smith, <u>Texas Agricultural
Experiment Station Bulletin No. 1555</u> (1987) (hereinafter
5   "Summers and Smith").

Prior to inserting the DNA sequence encoding the protein
into the baculovirus genome, the above described components,
comprising a promoter, leader (if desired), coding sequence
10 of interest, and transcription termination sequence, are
usually assembled into an intermediate transplacement
construct (transfer vector). This construct may contain a
single gene and operably linked regulatory elements;
multiple genes, each with its owned set of operably linked
15 regulatory elements; or multiple genes, regulated by the
same set of regulatory elements. Intermediate transplacement
constructs are often maintained in a replicon, such as an
extrachromosomal element (<i>eg.</i>, plasmids) capable of stable
maintenance in a host, such as a bacterium. The replicon
20 will have a replication system, thus allowing it to be
maintained in a suitable host for cloning and amplification.

Currently, the most commonly used transfer vector for
introducing foreign genes into AcNPV is pAc373. Many other
25 vectors, known to those of skill in the art, have also been
designed. These include, for example, pVL985 (which alters
the polyhedrin start codon from ATG to ATT, and which
introduces a BamHI cloning site 32 basepairs downstream from
the ATT; see Luckow and Summers, <u>Virology</u> (1989) <u>17</u>:31.
30

The plasmid usually also contains the polyhedrin
polyadenylation signal (Miller et al. (1988) <u>Ann. Rev.
Microbiol.</u>, <u>42</u>:177) and a procaryotic ampicillin-resistance
(<u>amp</u>) gene and origin of replication for selection and
35 propagation in <i>E. coli</i>.

Baculovirus transfer vectors usually contain a baculovirus
promoter. A baculovirus promoter is any DNA sequence capable

of binding a baculovirus RNA polymerase and initiating the
downstream (5' to 3') transcription of a coding sequence
(eg. structural gene) into mRNA. A promoter will have a
transcription initiation region which is usually placed
5   proximal to the 5' end of the coding sequence. This
transcription initiation region usually includes an RNA
polymerase binding site and a transcription initiation site.
A baculovirus transfer vector may also have a second domain
called an enhancer, which, if present, is usually distal to
10   the structural gene. Expression may be either regulated or
constitutive.

Structural genes, abundantly transcribed at late times in a
viral infection cycle, provide particularly useful promoter
15   sequences. Examples include sequences derived from the gene
encoding the viral polyhedron protein, Friesen et al.,
(1986) "The Regulation of Baculovirus Gene Expression," in:
The Molecular Biology of Baculoviruses (ed. Walter
Doerfler); EPO Publ. Nos. 127 839 and 155 476; and the gene
20   encoding the p10 protein, Vlak et al., (1988), J. Gen.
Virol. 69:765.

DNA encoding suitable signal sequences can be derived from
genes for secreted insect or baculovirus proteins, such as
25   the baculovirus polyhedrin gene (Carbonell et al. (1988)
Gene, 73:409). Alternatively, since the signals for
mammalian cell posttranslational modifications (such as
signal peptide cleavage, proteolytic cleavage, and
phosphorylation) appear to be recognized by insect cells,
30   and the signals required for secretion and nuclear
accumulation also appear to be conserved between the
invertebrate cells and vertebrate cells, leaders of non-
insect origin, such as those derived from genes encoding
human α-interferon, Maeda et al., (1985), Nature 315:592;
35   human gastrin-releasing peptide, Lebacq-Verheyden et al.,
(1988), Molec. Cell. Biol. 8:3129; human IL-2, Smith et al.,
(1985) Proc. Nat'l Acad. Sci. USA, 82:8404; mouse IL-3,
(Miyajima et al., (1987) Gene 58:273; and human

glucocerebrosidase, Martin et al. (1988) DNA, 7:99, can also
be used to provide for secretion in insects.

A recombinant polypeptide or polyprotein may be expressed
5   intracellularly or, if it is expressed with the proper
regulatory sequences, it can be secreted. Good intracellular
expression of nonfused foreign proteins usually requires
heterologous genes that ideally have a short leader sequence
containing suitable translation initiation signals preceding
10   an ATG start signal. If desired, methionine at the N-
terminus may be cleaved from the mature protein by in vitro
incubation with cyanogen bromide.

Alternatively, recombinant polyproteins or proteins which
15   are not naturally secreted can be secreted from the insect
cell by creating chimeric DNA molecules that encode a fusion
protein comprised of a leader sequence fragment that
provides for secretion of the foreign protein in insects.
The leader sequence fragment usually encodes a signal
20   peptide comprised of hydrophobic amino acids which direct
the translocation of the protein into the endoplasmic
reticulum.

After insertion of the DNA sequence and/or the gene encoding
25   the expression product precursor of the protein, an insect
cell host is co-transformed with the heterologous DNA of the
transfer vector and the genomic DNA of wild type baculovirus
-- usually by co-transfection. The promoter and
transcription termination sequence of the construct will
30   usually comprise a 2-5kb section of the baculovirus genome.
Methods for introducing heterologous DNA into the desired
site in the baculovirus virus are known in the art. (See
Summers and Smith supra; Ju et al. (1987); Smith et al.,
Mol. Cell. Biol. (1983) 3:2156; and Luckow and Summers
35   (1989)). For example, the insertion can be into a gene such
as the polyhedrin gene, by homologous double crossover
recombination; insertion can also be into a restriction
enzyme site engineered into the desired baculovirus gene.

Miller et al., (1989), Bioessays 4:91.The DNA sequence, when
cloned in place of the polyhedrin gene in the expression
vector, is flanked both 5' and 3' by polyhedrin-specific
sequences and is positioned downstream of the polyhedrin
5   promoter.

The newly formed baculovirus expression vector is
subsequently packaged into an infectious recombinant
baculovirus.   Homologous   recombination   occurs   at   low
10 frequency (between about 1% and about 5%); thus, the
majority of the virus produced after cotransfection is still
wild-type virus. Therefore, a method is necessary to
identify recombinant viruses. An advantage of the expression
system is a visual screen allowing recombinant viruses to be
15 distinguished. The polyhedrin protein, which is produced by
the native virus, is produced at very high levels in the
nuclei of infected cells at late times after viral
infection. Accumulated polyhedrin protein forms occlusion
bodies that also contain embedded particles. These occlusion
20 bodies, up to 15 μm in size, are highly refractile, giving
them a bright shiny appearance that is readily visualized
under the light microscope. Cells infected with recombinant
viruses lack occlusion bodies. To distinguish recombinant
virus from wild-type virus, the transfection supernatant is
25 plaqued onto a monolayer of insect cells by techniques known
to those skilled in the art. Namely, the plaques are
screened under the light microscope for the presence
(indicative of wild-type virus) or absence (indicative of
recombinant virus) of occlusion bodies. "Current Protocols
30 in Microbiology" Vol. 2 (Ausubel et al. eds) at 16.8 (Supp.
10, 1990); Summers and Smith, supra; Miller et al. (1989).

Recombinant baculovirus expression vectors have been
developed for infection into several insect cells. For
35 example, recombinant baculoviruses have been developed for,
inter alia: Aedes aegypti , Autographa californica, Bombyx
mori, Drosophila melanogaster, Spodoptera frugiperda, and
Trichoplusia ni (PCT Pub. No. WO 89/046699; Carbonell et

al., (1985) _J. Virol._ _56_:153; Wright (1986) _Nature_ _321_:718;
Smith et al., (1983) _Mol. Cell. Biol._ _3_:2156; and see
generally, Fraser, _et al._ (1989) _In Vitro Cell. Dev. Biol._
_25_:225).

5

Cells and cell culture media are commercially available for
both direct and fusion expression of heterologous
polypeptides in a baculovirus/expression system; cell
culture technology is generally known to those skilled in
10 the art. _See_, _eg._, Summers and Smith [_supra_].

The modified insect cells may then be grown in an
appropriate nutrient medium, which allows for stable
maintenance of the plasmid(s) present in the modified insect
15 host. Where the expression product gene is under inducible
control, the host may be grown to high density, and
expression induced. Alternatively, where expression is
constitutive, the product will be continuously expressed
into the medium and the nutrient medium must be continuously
20 circulated, while removing the product of interest and
augmenting depleted nutrients. The product may be purified
by such techniques as chromatography, _eg._, HPLC, affinity
chromatography, ion exchange chromatography, etc.;
electrophoresis; density gradient centrifugation; solvent
25 extraction, or the like. As appropriate, the product may be
further purified, as required, so as to remove substantially
any insect proteins which are also secreted in the medium or
result from lysis of insect cells, so as to provide a
product which is at least substantially free of host debris,
30 _eg._, proteins, lipids and polysaccharides.

In order to obtain protein expression, recombinant host
cells derived from the transformants are incubated under
conditions which allow expression of the recombinant protein
35 encoding sequence. These conditions will vary, dependent
upon the host cell selected. However, the conditions are
readily ascertainable to those of ordinary skill in the art,
based upon what is known in the art.

iii. Bacterial Systems

Bacterial expression techniques are known in the art. A
bacterial promoter is any DNA sequence capable of binding
5   bacterial RNA polymerase and initiating the downstream (3")
transcription of a coding sequence (eg. structural gene)
into mRNA. A promoter will have a transcription initiation
region which is usually placed proximal to the 5' end of the
coding sequence. This transcription initiation region
10 usually includes an RNA polymerase binding site and a
transcription initiation site. A bacterial promoter may also
have a second domain called an operator, that may overlap an
adjacent RNA polymerase binding site at which RNA synthesis
begins. The operator permits negative regulated (inducible)
15 transcription, as a gene repressor protein may bind the
operator and thereby inhibit transcription of a specific
gene. Constitutive expression may occur in the absence of
negative regulatory elements, such as the operator. In
addition, positive regulation may be achieved by a gene
20 activator protein binding sequence, which, if present is
usually proximal (5') to the RNA polymerase binding
sequence. An example of a gene activator protein is the
catabolite activator protein (CAP), which helps initiate
transcription of the lac operon in Escherichia coli (E.
25 coli) [Raibaud et al. (1984) Annu. Rev. Genet. 18:173].
Regulated expression may therefore be either positive or
negative, thereby either enhancing or reducing
transcription.

30 Sequences encoding metabolic pathway enzymes provide
particularly useful promoter sequences. Examples include
promoter sequences derived from sugar metabolizing enzymes,
such as galactose, lactose (lac) [Chang et al. (1977) Nature
198:1056], and maltose. Additional examples include promoter
35 sequences derived from biosynthetic enzymes such as
tryptophan (trp) [Goeddel et al. (1980) Nuc. Acids Res.
8:4057; Yelverton et al. (1981) Nucl. Acids Res. 9:731; U.S.
Patent No. 4,738,921; EPO Publ. Nos. 036 776 and 121 775].

The g-laotamase (bla) promoter system [Weissmann (1981) "The cloning of interferon and other mistakes." In Interferon 3 (ed. I. Gresser)], bacteriophage lambda PL [Shimatake et al. (1981) Nature 292:128] and T5 [U.S. Patent No. 4,689,406]
5 promoter systems also provide useful promoter sequences.

In addition, synthetic promoters which do not occur in nature also function as bacterial promoters. For example, transcription activation sequences of one bacterial or
10 bacteriophage promoter may be joined with the operon sequences of another bacterial or bacteriophage promoter, creating a synthetic hybrid promoter [U.S. Patent No. 4,551,433]. For example, the tac promoter is a hybrid trp-lac promoter comprised of both trp promoter and lac
15 operon sequences that is regulated by the lac repressor [Amann et al. (1983) Gene 25:167; de Boer et al. (1983) Proc. Natl. Acad. Sci. 80:21]. Furthermore, a bacterial promoter can include naturally occurring promoters of non-bacterial origin that have the ability to bind bacterial RNA
20 polymerase and initiate transcription. A naturally occurring promoter of non-bacterial origin can also be coupled with a compatible RNA polymerase to produce high levels of expression of some genes in prokaryotes. The bacteriophase T7 RNA polymerase/promoter system is an example of a coupled
25 promoter system [Studier et al. (1986) J. Mol. Biol. 189:113; Tabor et al. (1985) Proc Natl. Acad. Sci. 82:1074]. In addition, a hybrid promoter can also be comprised of a bacteriophage promoter and an E. coli operator region (EPO Publ. No. 267 851).
30

In addition to a functioning promoter sequence, an efficient ribosome binding site is also useful for the expression of foreign genes in prokaryotes. In E. coli, the ribosome binding site is called the Shine-Dalgarno (SD) sequence and
35 includes an initiation codon (ATG) and a sequence 3-9 nucleotides in length located 3-11 nucleotides upstream of the initiation codon [Shine et al. (1975) Nature 254:34]. The SD sequence is thought to promote binding of mRNA to the

ribosome by the pairing of bases between the SD sequence and
the 3' and of E. coli 16S rRNA [Steitz et al. (1979)
"Genetic signals and nucleotide sequences in messenger RNA."
In Biological Regulation and Development: Gene Expression
5 (ed. R.F. Goldberger)]. To express eukaryotic genes and
prokaryotic genes with weak ribosome-binding site [Sambrook
et al. (1989) "Expression of cloned genes in Escherichia
coli." In Molecular Cloning: A Laboratory Manual].

10 A DNA molecule may be expressed intracellularly. A promoter
sequence may be directly linked with the DNA molecule, in
which case the first amino acid at the N-terminus will
always be a methionine, which is encoded by the ATG start
codon. If desired, methionine at the N-terminus may be
15 cleaved from the protein by in vitro incubation with
cyanogen bromide or by either in vivo on in vitro incubation
with a bacterial methionine N-terminal peptidase (EPO Publ.
No. 219 237).

20 Fusion proteins provide an alternative to direct expression.
Usually, a DNA sequence encoding the N-terminal portion of
an endogenous bacterial protein, or other stable protein, is
fused to the 5' end of heterologous coding sequences. Upon
expression, this construct will provide a fusion of the two
25 amino acid sequences. For example, the bacteriophage lambda
cell gene can be linked at the 5' terminus of a foreign gene
and expressed in bacteria. The resulting fusion protein
preferably retains a site for a processing enzyme (factor
Xa) to cleave the bacteriophage protein from the foreign
30 gene [Nagai et al. (1984) Nature 309:810]. Fusion proteins
can also be made with sequences from the lacZ [Jia et al.
(1987) Gene 60:197], trpE [Allen et al. (1987) J.
Biotechnol. 5:93; Makoff et al. (1989) J. Gen. Microbiol.
135:11], and Chey [EPO Publ. No. 324 647] genes. The DNA
35 sequence at the junction of the two amino acid sequences may
or may not encode a cleavable site. Another example is a
ubiquitin fusion protein. Such a fusion protein is made with
the ubiquitin region that preferably retains a site for a

processing enzyme (eg. ubiquitin specific processing-protease) to cleave the ubiquitin from the foreign protein. Through this method, native foreign protein can be isolated [Miller et al. (1989) Bio/Technology 7:698].

5

Alternatively, foreign proteins can also be secreted from the cell by creating chimeric DNA molecules that encode a fusion protein comprised of a signal peptide sequence fragment that provides for secretion of the foreign protein

10 in bacteria [U.S. Patent No. 4,336,336]. The signal sequence fragment usually encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell. The protein is either secreted into the growth media (gram-positive bacteria) or into the

15 periplasmic space, located between the inner and outer membrane of the cell (gram-negative bacteria). Preferably there are processing sites, which can be cleaved either in vivo or in vitro encoded between the signal peptide fragment and the foreign gene.

20

DNA encoding suitable signal sequences can be derived from genes for secreted bacterial proteins, such as the E. coli outer membrane protein gene (ompA) [Masui et al. (1983), in: Experimental Manipulation of Gene Expression; Ghrayeb et al.

25 (1984) EMBO J. 3:2437] and the E. coli alkaline phosphatase signal sequence (phoA) [Oka et al. (1985) Proc. Natl. Acad. Sci. 82:7212]. As an additional example, the signal sequence of the alpha-amylase gene from various Bacillus strains can be used to secrete heterologous proteins from B. subtilis

30 [Palva et al. (1982) Proc. Natl. Acad. Sci. USA 79:5582; EPO Publ. No. 244 042].

Usually, transcription termination sequences recognized by bacteria are regulatory regions located 3' to the

35 translation stop codon, and thus together with the promoter flank the coding sequence. These sequences direct the transcription of an mRNA which can be translated into the polypeptide encoded by the DNA. Transcription termination

sequences frequently include DNA sequences of about 50 nucleotides capable of forming stem loop structures that aid in terminating transcription. Examples include transcription termination sequences derived from genes with strong

5   promoters, such as the trp gene in E. coli as well as other biosynthetic genes.

Usually, the above described components, comprising a promoter, signal sequence (if desired), coding sequence of

10   interest, and transcription termination sequence, are put together into expression constructs. Expression constructs are often maintained in a replicon, such as an extrachromosomal element (eg., plasmids) capable of stable maintenance in a host, such as bacteria. The replicon will

15   have a replication system, thus allowing it to be maintained in a procaryotic host either for expression or for cloning and amplification. In addition, a replicon may be either a high or low copy number plasmid. A high copy number plasmid will generally have a copy number ranging from about 5 to

20   about 200, and usually about 10 to about 150. A host containing a high copy number plasmid will preferably contain at least about 10, and more preferably at least about 20 plasmids. Either a high or low copy number vector may be selected, depending upon the effect of the vector and

25   the foreign protein on the host.

Alternatively, the expression constructs can be integrated into the bacterial genome with an integrating vector. Integrating vectors usually contain at least one sequence

30   homologous to the bacterial chromosome that allows the vector to integrate. Integrations appear to result from recombinations between homologous DNA in the vector and the bacterial chromosome. For example, integrating vectors constructed with DNA from various Bacillus strains integrate

35   into the Bacillus chromosome (EPO Publ. No. 127 328). Integrating vectors may also be comprised of bacteriophage or transposon sequences.

Usually, extrachromosomal and integrating expression
constructs may contain selectable markers to allow for the
selection of bacterial strains that have been transformed.
Selectable markers can be expressed in the bacterial host

5   and may include genes which render bacteria resistant to
drugs such as ampicillin, chloramphenicol, erythromycin,
kanamycin (neomycin), and tetracycline [Davies et al. (1978)
Annu. Rev.Microbiol. 32:469]. Selectable markers may also
include biosynthetic genes, such as those in the histidine,

10   tryptophan, and leucine biosynthetic pathways.

Alternatively, some of the above described components can be
put together in transformation vectors. Transformation
vectors are usually comprised of a selectable market that is

15   either maintained in a replicon or developed into an
integrating vector, as described above.

Expression and transformation vectors, either extra-
chromosomal replicons or integrating vectors, have been

20   developed for transformation into many bacteria. For
example, expression vectors have been developed for, inter
alia, the following bacteria: Bacillus subtilis [Palva et
al. (1982) Proc. Natl. Acad. Sci. USA 79:5582; EPO Publ.
Nos. 036 259 and 063 953; PCT Publ. No. WO 84/04541],

25   Escherichia coli [Shimatake et al. (1981) Nature 292:128;
Amann et al. (1985) Gene 40:183; Studier et al. (1986) J.
Mol. Biol. 189:113; EPO Publ. Nos. 036 776, 136 829 and 136
907], Streptococcus cremoris [Powell et al. (1988) Appl.
Environ. Microbiol. 54:655]; Streptococcus lividans [Powell

30   et al. (1988) Appl. Environ. Microbiol. 54:655],
Streptomyces lividans [U.S. Patent No. 4,745,056].

Methods of introducing exogenous DNA into bacterial hosts
are well-known in the art, and usually include either the

35   transformation of bacteria treated with $CaCl_2$ or other
agents, such as divalent cations and DMSO. DNA can also be
introduced into bacterial cells by electroporation.
Transformation procedures usually vary with the bacterial

species to be transformed. See *eg.*, [Masson et al. (1989) FEMS Microbiol. Lett. 60:273; Palva et al. (1982) Proc. Natl. Acad. Sci. USA 79:5582; EPO Publ. Nos. 036 259 and 063 953; PCT Publ. No. WO 84/04541, Bacillus], [Miller et al.

5 (1988) Proc. Natl. Acad. Sci. 85:856; Wang et al. (1990) J. Bacteriol. 172:949, Campylobacter], [Cohen et al. (1973) Proc. Natl. Acad. Sci. 69:2110; Dower et al. (1988) Nucleic Acids Res. 16:6127; Kushner (1978) "An improved method for transformation of Escherichia coli with ColE1-derived

10 plasmids. In Genetic Engineering: Proceedings of the International Symposium on Genetic Engineering (eds. H.W. Boyer and S. Nicosia); Mandel et al. (1970) J. Mol. Biol. 53:159; Taketo (1988) Biochim. Biophys. Acta 949:318; Escherichia], [Chassy et al. (1987) FEMS Microbiol. Lett.

15 44:173 Lactobacillus]; [Fiedler et al. (1988) Anal. Biochem 170:38, Pseudomonas]; [Augustin et al. (1990) FEMS Microbiol. Lett. 66:203, Staphylococcus], [Barany et al. (1980) J. Bacteriol. 144:698; Harlander (1987) "Transformation of Streptococcus lactis by electroporation,

20 in: Streptococcal Genetics (ed. J. Ferretti and R. Curtiss III); Perry et al. (1981) Infec. Immun. 32:1295; Powell et al. (1988) Appl. Environ. Microbiol. 54:655; Somkuti et al. (1987) Proc. 4th Evr. Conq. Biotechnology 1:412, Streptococcus].

25

iv. Yeast Expression

Yeast expression systems are also known to one of ordinary skill in the art. A yeast promoter is any DNA sequence

30 capable of binding yeast RNA polymerase and initiating the downstream (3') transcription of a coding sequence (*eg.* structural gene) into mRNA. A promoter will have a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This

35 transcription initiation region usually includes an RNA polymerase binding site (the "TATA Box") and a transcription initiation site. A yeast promoter may also have a second domain called an upstream activator sequence (UAS), which,

if present, is usually distal to the structural gene. The
UAS permits regulated (inducible) expression. Constitutive
expression occurs in the absence of a UAS. Regulated
expression may be either positive or negative, thereby
5   either enhancing or reducing transcription.

Yeast is a fermenting organism with an active metabolic
pathway, therefore sequences encoding enzymes in the
metabolic pathway provide particularly useful promoter
10 sequences. Examples include alcohol dehydrogenase (ADH) (EPO
Publ. No. 284 044), enolase, glucokinase, glucose-6-
phosphate   isomerase,   glyceraldehyde-3-phosphate-
dehydrogenase   (GAP   or   GAPDH),   hexokinase,
phosphofructokinase, 3-phosphoglycerate mutase, and pyruvate
15 kinase (PyK) (EPO Publ. No. 329 203). The yeast PHO5 gene,
encoding acid phosphatase, also provides useful promoter
sequences [Myanohara et al. (1983) Proc. Natl. Acad. Sci.
USA 80:1].

20 In addition, synthetic promoters which do not occur in
nature also function as yeast promoters. For example, UAS
sequences of one yeast promoter may be joined with the
transcription activation region of another yeast promoter,
creating a synthetic hybrid promoter. Examples of such
25 hybrid promoters include the ADH regulatory sequence linked
to the GAP transcription activation region (U.S. Patent Nos.
4,876,197 and 4,880,734). Other examples of hybrid promoters
include promoters which consist of the regulatory sequences
of either the ADH2, GAL4, GAL10, OR PHO5 genes, combined
30 with the transcriptional activation region of a glycolytic
enzyme gene such as GAP or PyK (EPO Publ. No. 164 556).
Furthermore, a yeast promoter can include naturally
occurring promoters of non-yeast origin that have the
ability to bind yeast RNA polymerase and initiate
35 transcription. Examples of such promoters include, inter
alia, [Cohen et al. (1980) Proc. Natl. Acad. Sci. USA
77:1078; Henikoff et al. (1981) Nature 283:835; Hollenberg
et al. (1981) Curr. Topics Microbiol. Immunol. 96:119;

Hollenberg <u>et al</u>. (1979) "The Expression of Bacterial Antibiotic Resistance Genes i the Yeast Saccharomyces cerevisiae," in: <u>Plasmids of Medical, Environmental and Commercial Importance</u> (eds. K>N> Timmis and A. Puhler);
5 Mercerau-Puigalon <u>et al</u>. (1980) <u>Gene 11</u>:163; Panthier <u>et al</u>. (1980) <u>Curr. Genet. 2</u>:109;].

A DNA molecule may be expressed intracellularly in yeast. A promoter sequence may be directly linked with the DNA
10 molecule, in which case the first amino acid at the N-terminus of the recombinant protein will always be a methionine, which is encoded by the ATG start codon. If desired, methionine at the N-terminus may be cleaved from the protein by <u>in vitro</u> incubation with cyanogen bromide.
15

Fusion proteins provide an alternative for yeast expression systems, as well as in mammalian, baculovirus, and bacterial expression systems. Usually, a DNA sequence encoding the N-terminal portion of an endogenous yeast protein, or other
20 stable protein, is fused to the 5' end of heterologous coding sequences. Upon expression, this construct will provide a fusion of the two amino acid sequences. For example, the yeast or human superoxide dismutase (SOD) gene, can be linked at the 5' terminus of a foreign gene and
25 expressed in yeast. The DNA sequence at the junction of the two amino acid sequences may or may not encode a cleavable site. See eg., EPO Publ. No. 196 056. Another example is a ubiquitin fusion protein. Such a fusion protein is made with the ubiquitin region that preferably retains a site for a
30 processing enzyme (eg. ubiquitin-specific processing protease) to cleave the ubiquitin from the foreign protein. Through this method, therefore, native foreign protein can be isolated (see, eg., PCT Publ. No. WO 88/024066).

35 Alternatively, foreign proteins can also be secreted from the cell into the growth media by creating chimeric DNA molecules that encode a fusion protein comprised of a leader sequence fragment that provide for secretion in yeast of the

38

foreign protein. Preferably, there are processing sites encoded between the leader fragment and the foreign gene that can be cleaved either in vivo or in vitro. The leader sequence fragment usually encodes a signal peptide comprised

5   of hydrophobic amino acids which direct the secretion of the protein from the cell.

DNA encoding suitable signal sequences can be derived from genes for secreted yeast proteins, such as the yeast

10   invertase gene (EPO Publ. No. 012 873; JPO Publ. No. 62,096,086) and the A-factor gene (U.S. Patent No. 4,588,684). Alternatively, leaders of non-yeast origin, such as an interferon leader, exist that also provide for secretion in yeast (EPO Publ. No. 060 057).

15

A preferred class of secretion leaders are those that employ a fragment of the yeast alpha-factor gene, which contains both a "pre" signal sequence, and a "pro" region. The types of alpha-factor fragments that can be employed include the

20   full-length pre-pro alpha factor leader (about 83 amino acid residues) as well as truncated alpha-factor leaders (usually about 25 to about 50 amino acid residues) (U.S. Patent Nos. 4,546,083 and 4,870,008; EPO Publ. No. 324 274). Additional leaders employing an alpha-factor leader fragment that

25   provides for secretion include hybrid alpha-factor leaders made with a presequence of a first yeast, but a pro-region from a second yeast alphafactor. (See eg., PCT Publ. No. WO 89/02463.)

30   Usually, transcription termination sequences recognized by yeast are regulatory regions located 3' to the translation stop codon, and thus together with the promoter flank the coding sequence. These sequences direct the transcription of an mRNA which can be translated into the polypeptide encoded

35   by the DNA. Examples of transcription terminator sequence and other yeast-recognized termination sequences, such as those coding for glycolytic enzymes.

Usually, the above described components, comprising a
promoter, leader (if desired), coding sequence of interest,
and transcription termination sequence, are put together
into expression constructs. Expression constructs are often
5   maintained in a replicon, such as an extrachromosomal
element (eg., plasmids) capable of stable maintenance in a
host, such as yeast or bacteria. The replicon may have two
replication systems, thus allowing it to be maintained, for
example, in yeast for expression and in a procaryotic host
10   for cloning and amplification. Examples of such yeast-
bacteria shuttle vectors include YEp24 [Botstein et al.
(1979) Gene 8:17-24], pCl/1 [Brake et al. (1984) Proc. Natl.
Acad. Sci USA 81:4642-4646], and YRp17 [Stinchcomb et al.
(1982) J. Mol. Biol. 158:157]. In addition, a replicon may
15   be either a high or low copy number plasmid. A high copy
number plasmid will generally have a copy number ranging
from about 5 to about 200, and usually about 10 to about
150. A host containing a high copy number plasmid will
preferably have at least about 10, and more preferably at
20   least about 20. Enter a high or low copy number vector may
be selected, depending upon the effect of the vector and the
foreign protein on the host. See eg., Brake et al., supra.

Alternatively, the expression constructs can be integrated
25   into the yeast genome with an integrating vector.
Integrating vectors usually contain at least one sequence
homologous to a yeast chromosome that allows the vector to
integrate, and preferably contain two homologous sequences
flanking the expression construct. Integrations appear to
30   result from recombinations between homologous DNA in the
vector and the yeast chromosome [Orr-Weaver et al. (1983)
Methods in Enzymol. 101:228-245]. An integrating vector may
be directed to a specific locus in yeast by selecting the
appropriate homologous sequence for inclusion in the vector.
35   See Orr-Weaver et al., supra. One or more expression
construct may integrate, possibly affecting levels of
recombinant protein produced [Rine et al. (1983) Proc. Natl.
Acad. Sci. USA 80:6750]. The chromosomal sequences included

in the vector can occur either as a single segment in the
vector, which results in the integration of the entire
vector, or two segments homologous to adjacent segments in
the chromosome and flanking the expression construct in the
5   vector, which can result in the stable integration of only
the expression construct.

Usually, extrachromosomal and integrating expression
constructs may contain selectable markers to allow for the
10 selection of yeast strains that have been transformed.
Selectable markers may include biosynthetic genes that can
be expressed in the yeast host, such as ADE2, HIS4, LEU2,
TRP1, and ALG7, and the G418 resistance gene, which confer
resistance in yeast cells to tunicamycin and G418,
15 respectively. In addition, a suitable selectable marker may
also provide yeast with the ability to grow in the presence
of toxic compounds, such as metal. For example, the presence
of CUP1 allows yeast to grow in the presence of copper ions
[Butt et al. (1987) Microbiol, Rev. 51:351].
20

Alternatively, some of the above described components can be
put together into transformation vectors. Transformation
vectors are usually comprised of a selectable marker that is
either maintained in a replicon or developed into an
25 integrating vector, as described above.

Expression and transformation vectors, either
extrachromosomal replicons or integrating vectors, have been
developed for transformation into many yeasts. For example,
30 expression vectors have been developed for, inter alia, the
following yeasts:Candida albicans [Kurtz, et al. (1986) Mol.
Cell. Biol. 6:142], Candida maltose [Kunze, et al. (1985) J.
Basic Microbiol. 25:141]. Hansenula polymorpha [Gleeson, et
al. (1986) J. Gen. Microbiol. 132:3459; Roggenkamp et al.
35 (1986) Mol. Gen. Genet. 202:302], Kluyveromyces fragilis
[Das, et al. (1984) J. Bacteriol. 158:1165], Kluyveromyces
lactis [De Louvencourt et al. (1983) J. Bacteriol. 154:737;
Van den Berg et al. (1990) Bio/Technology 8:135], Pichia

guillerimondii [Kunze et al. (1985) J. Basic Microbiol.
25:141], Pichia pastoris [Cregg, et al. (1985) Mol. Cell.
Biol. 5:3376; U.S. Patent Nos. 4,837,148 and 4,929,555],
Saccharomyces cerevisiae [Hinnen et al. (1978) Proc. Natl.
5 Acad. Sci. USA 75:1929; Ito et al. (1983) J. Bacteriol.
153:163], Schizosaccharomyces pombe [Beach and Nurse (1981)
Nature 300:706], and Yarrowia lipolytica [Davidow, et al.
(1985) Curr. Genet. 10:380471 Gaillardin, et al. (1985)
Curr. Genet. 10:49].

10

Methods of introducing exogenous DNA into yeast hosts are
well-known in the art, and usually include either the
transformation of spheroplasts or of intact yeast cells
treated with alkali cations. Transformation procedures
15 usually vary with the yeast species to be transformed. See
eg., [Kurtz et al. (1986) Mol. Cell. Biol. 6:142; Kunze et
al. (1985) J. Basic Microbiol. 25:141; Candida]; [Gleeson et
al. (1986) J. Gen. Microbiol. 132:3459; Roggenkamp et al.
(1986) Mol. Gen. Genet. 202:302; Hansenula]; [Das et al.
20 (1984) J. Bacteriol. 158:1165; De Louvencourt et al. (1983)
J. Bacteriol. 154:1165; Van den Berg et al. (1990)
Bio/Technology 8:135; Kluyveromyces]; [Cregg et al. (1985)
Mol. Cell. Biol. 5:3376; Kunze et al. (1985) J. Basic
Microbiol. 25:141; U.S. Patent Nos. 4,837,148 and 4,929,555;
25 Pichia]; [Hinnen et al. (1978) Proc. Natl. Acad. Sci. USA
75;1929; Ito et al. (1983) J. Bacteriol. 153:163
Saccharomyces]; [Beach and Nurse (1981) Nature 300:706;
Schizosaccharomyces]; [Davidow et al. (1985) Curr. Genet.
10:39; Gaillardin et al. (1985) Curr. Genet. 10:49;
30 Yarrowia].

1.   Preparation of LT-A72R mutant

a.   Sourc   f LT DNA
35 The 1.5 kb SmaI-EcoRI fragment from plasmid pEWD299,
containing the LT-A gene and the LTpromoter region [Pronk et
al. (1985) J. Biol. Chem. 260:13580; Spicer et al. (1981)
Proc. Natl. Acad. Sci. USA 78:50] was subcloned into the

Bluescript KS vector (Stratagene). The resultant vector, termed BS-LT-A, was used for site-directed mutagenesis [Zoller & Smith (1982) *Nucl. Acid. Res.* **10**:6487)].

5 b.   **Methods of mutation**
Site-directed mutagenesis was performed according to the method of Zoller & Smith [*supra*] on single-stranded DNA of BS-LT-A vector. The oligonucleotide used:
     $^{5'}$GCTCACTTACGTGGACAGTCT$^{3'}$ (oligoLT-A72R)
10 mutates the codon for Ala-72 (GCA) to an Arg codon (CGT).

The mutated *SmaI-EcoRI* fragment containing the mutated codon was ligated with the 0.57 kb *EcoRI-HindIII* fragment [Pronk *et al.*; Spicer *et al.*] containing the gene coding for LT-B
15 and cloned into *Bluescript KS* vector (Stratagene) to generate vector BS-LTA72R.

c. **Expression and purification of the LT-A72R mutant.**
E.coli were transformed with BS-LTA72R vector and grown in
20 LB broth in a 5 litre fermenter. The mutant protein LT-A72R was purified from the periplasm [Pronk *et al.*; Magagnoli *et al.* (1996) *Infect Immun* 64:5434] using controlled pore glass (CPG 350, Serva) and A5M Agarose columns and then by gel filtration using Sephacryl S-200™.
25

An aliquot of the purified material was analysed on a Superdex 200HR column. Figure 1 shows the elution profile of LT-A72R in comparison with wild-type LT and mutant LTK63 (Ser-63-Lys mutant – see WO93/13202 and also Pizza *et al.*
30 (1994) *J.Exp. Med.* 6:2147).

LT-A72R elutes as a single peak corresponding to the fully-assembled holotoxin, with a profile identical to that of wild-type LT and LTK63. This demonstrates that the A72R
35 mutation does not alter the structure of the A subunit, that it is efficiently exported to the periplasm, and correctly assembled as AB$_5$. 100μl aliquots of A72R stored at 4 C were analysed in the same way every two months for a y ar and no

change in elution profile was detected.

2. Physical characterisation

5 **a. Trypsin digestion**

45μg toxin (LT, LT-A72R, LTK63) was treated with 9μg trypsin
in a final volume of 150μl 10mM Tris, pH 7.5, at 37°C. 30μl
samples were collected after 5 and 30 minutes incubation and
the reaction was stopped with 3.6μg trypsin inhibitor. 10μl
10 4xconcentrated electrophoresis sample buffer was added to
each sample and the mixture was heated to 95°C for 10
minutes. Proteins were loaded onto 15% SDS minigels and
either stained with Coomassie brilliant blue R-250 or
transferred onto a nitrocellulose membrane, which was
15 subsequently incubated with a rabbit anti-LT polyclonal
serum at a dilution of 1:300.

Figure 2 shows the results of this Western blot analysis.
After 5 minutes incubation, trypsin causes almost immediate
20 nicking of the A subunit into A1 and A2 domains, and nicking
is complete by 30 minutes. No sensitivity differences were
detected between LT, LT-A72R, or LTK63.

b. ADP-ribosylation of polyarginine
25 LT (wild-type and mutant) was analysed as described by Lai
et al. [*BBRC* 102:1021, 1981] and the results are shown in
Figure 3.

The ADP-ribosylation activity of LT-A72R is at least 2
30 orders of magnitude lower than that of wild-type LT: the
minimal concentration to obtain a detectable activity was
0.5μg for LT, and 85μg for LT-A72R. In contrast, LTK63 is
totally devoid of activity.

35 **c. Toxicity**

In vitro toxicity was assessed by following the
morphological changes caused by LT on Y1 adrenal cells
[Donta *et al.* (1973) *Science* 183:334]. The assay was

performed in microtitre plates using 50000 cells/well with
twofold dilutions of LT, LT-A72R and LTK63, starting from
80pg/well for wild-type and 40μg/well for mutants.
Morphological changes were read after 48 hours incubation.

5

In vivo toxicity was assessed using an ileal loop assay [De
(1959) *Nature* 183:1533]. Two New Zealand adult rabbits (*ca.*
2.5 kg) were used for each assay and 1ml samples of toxin
(wild-type, LT-A72R, LTK63) were injected into each loop at
10 various concentrations, with a control loop receiving 1ml
PBS. After 18-20 hours, the volume of liquid accumulated in
each loop was measured with a syringe and the length of each
loop was measured again. The results, from 4-6 repeats,
expressed as volume of liquid per unit length of the loop
15 (ml/cm) are shown in Figure 5.

In agreement with the ADP-ribosylation results, LT-A72R had
10^{-5} toxicity of wild-type LT on Y1 cells (Figure 4) and was
approximately 20x less toxic than wild-type in the ileal
20 loop assay (Figure 5). As previously reported, LTK63 was
completely non-toxic by both assays.

3. Immunological characterisation

25 a. Mucosal adjuvanticity

The adjuvanticity of LT-A72R was tested using the protocol
of Douce *et al.* [*PNAS* 92:1644-1648 (1995)]. Groups of ten
BALB/c mice (female, 4-6 weeks old) were immunised
intranasally with 1μg toxin (LT, LT-A72R, LTB, or LK-K63)
30 and 10μg OVA. The animals were lightly anaesthetised and
immunised on day 0, 21 and 35 with a 15μl volume per
nostrel. Immune responses were followed in serum samples
taken on days 0, 20, 34 and 52. The animals were sacrificed
and nasal lavages were performed by repeated flushing and
35 aspiration of 1ml PBS containing 0.1% BSA.

LT-specific antibodies were measured using a GM1 capture
ELISA. Each well on 96-well plates was first coated with

150ng GM1 ganglioside by overnight incubation at 4°C. Wells
were then washed three times with PBS (+0.05% Tween-20) and
50ng toxin was added to each well. Plates were incubated at
2 hours at 37°C. OVA-specific antibodies were assessed by
5   coating each well with 60µg/ml OVA and incubated overnight
at 4°C.

The plates were washed and wells saturated with PBS (+1%
BSA) for 1 hour at 37°C. Sera from individual mice were
10   tested starting from a dilution of 1:50 in PBS; nasal
lavages were tested starting from undiluted. Plates were
incubated at 37°C for 2 hours. Specific Ig were measured
using horseradish peroxidase-conjugated rabbit anti-mouse
Ig.   Antibodies   were   then   revealed   by   adding
15   o-phenylenediamine as a substrate. After 10 minutes the
reaction was blocked by adding 12.5% H_2SO_4.

IgG subclasses were determined with IgG1, IgG2a, IgG2b, or
IgG3 biotinylated antibodies. Peroxidase-streptavadin was
20   then added to each well (1:1000) and the plates were
incubated at 37°C for 1 hour. Bound antibody was visualised
as above.

Absorbances were read at 490nm and ELISA titres were
25   determined arbitrarily as the reciprocal of the last
dilution which gave an $OD_{490} \geq 0.3$ above the level measured in
pre-immune sera.

Titres of specific IgA in the sera and in the mucosal
30   lavages were measured using biotin-conjugated goat
anti-mouse IgA α chain specific antibody, followed by
streptavadin-peroxidase. Bound antibodies were revealed
using the OPD substrate as described above. ELISA titres
were determined arbitrarily as the reciprocal of the last
35   dilution which gave an $OD_{490} \geq 0.2$ above that of the
non-immunised controls.

Values were always normalised using a positive control

sample in each plate.

As shown in Figure 6, wild-type LT and LT-A72R induced the highest anti-OVA antibody response. LTK63 induced an

5   intermediate level, and LTB gave a low response. The antigen-specific antibody response was detectable after a single immunisation for wild-type LT and the A72R mutant, whereas (as previously described) LTK63 required at least two immunisations to induce serum anti-OVA antibodies.

10

OVA-specific IgG isotypes were measured in pools of sera of the last bleeding (Figure 7). High titres of anti-OVA IgG1, IgG2a and IgG2b were induced in the groups receiving wild-type LT or the A72R mutant as an adjuvant, although the

15   dominant isotype with the mutant was IgG2a. OVA-specific IgG3 antibodies were never detectable. LTB was not a good adjuvant.

In all experiments, OVA-specific IgA was never detected

20   after just 1 or two intranasal immunisations. Following a third immunisation, however, a serum IgA response was detected with wild-type LT and with the A72R and K63 mutants, but not with LTB (Figure 8A). A similar pattern was seen at the mucosal level (Figure 8B), and after three

25   immunisations OVA-specific IgA was found in the nasal washes of the wild-type group and the A72R and K63 mutant groups, but not in the control or LTB groups.

Serum anti-LT antibody responses are shown in Figure 9. The

30   mice mounted responses after the first immunisation, which was significantly boosted by the second. The A72R mutant was more immunogenic that the non-toxic K63 mutant, and both were much more immunogenic than LTB.

35   **b. OVA-driven proliferative response**
14-20 days after 2 or 3 intranasal immunisations with OVA+LT (wild-type or mutant), 2 or 3 mice per group were sacrificed and spleens removed. Spleen cell suspensions were obtained

and resuspended in complete DMEM (10% FCS, 2mM L-glutamine, 15mM Hepes, 100U penicillin/streptomycin, 50mM 2-mercapto-ethanol). 2×10^5 spleen cells/well were seeded in U-bottomed 96-well plates and cultured in the presence of different

5 concentrations of OVA for 5 days. [^{3}H]-thymidine was added (1μCi/well) 16 hours before the end of culture. Cells were then harvested with a cell harvester, and [^{3}H]-thymidine incorporation was evaluated by liquid scintillation counting (Figure 10).

10

It is clear that intranasal co-administration of antigen with wild-type or with A72R or K63 mutants induced a priming of OVA-specific T cells *in vivo*, which was much stronger than that detectable after immunisation with OVA alone or

15 with OVA+LTB.

c. *In vivo* challenge

The LD_{50} for LT was determined by inoculating groups of 10 BALB/c mice (female, 9 weeks old) intraperitoneally with LT

20 (12.5μg, 25μg, 50μg, or 100μg) or PBS. After 7 days of observation, LD_{50} was determined as 20.4μg.

Four week old BALB/c mice were immunised intranasally at day 0 and 21 with 1μg toxin (LT, LT-A72R, LT-K63, or LTB) and

25 challenged with LT ($2 \times LD_{50}$) at day 35. They were observed for death for 7 days. Sera were collected at days 0, 20 and 35 and anti-LT titres analysed by ELISA (as above).

All mice immunised with wild-type or mutant LT survived to

30 the challenge, whereas only 30% of those receiving LTB survived. All mice in the control group died (Figure 11A).

Sera of mice immunised with wild-type LT, or with the A72R or K63 mutants, contained very high and comparable levels of

35 anti-LT antibodies (Figure 11B). In mice receiving LTB, however, the titres were 10-20x lower. The three LTB mice who survived to the LT challenge (open circles) had significantly higher titres than those in the dead animals.

4. Conclusions

ADP-ribosylation activity is not necessary for the adjuvanticity of LT, but the presence of low levels of
5 enzymatic activity may be useful to induce a faster and higher immune response to co-administered antigens.

The A72R mutant of LT is an effective mucosal adjuvant. In addition, the mutant retains the immunogenicity of wild-type
10 LT and is able to induce protective immunity against LT. It may therefore also be useful for anti-diarrhoea vaccination.

It will, of course, be understood that the invention is
15 described above by way of example only and modifications may be made within the scope and spirit of the invention.